Biochemical Pharmacology Vol 27 pp. 2879–2890.

0006-2952/78/1215-2879 $02.00/0

MECHANISMS OF THE METABOLIC DISTURBANCES CAUSED BY HYPOGLYCIN AND BY PENT-4-ENOIC ACID *IN VITRO* STUDIES

DAVID BILLINGTON*, HARALD OSMUNDSEN† and H. STANLEY A. SHERRATT

Department of Pharmacological Sciences, Medical School, University of Newcastle upon Tyne, Newcastle upon Tyne, NE1 7RU, U.K.

(*Received* 3 *March* 1978; *accepted* 18 *April* 1978)

Abstract—1. In the presence of the hypoglycin metabolites methylenecyclopropylpyruvate (MCPP) and methylenecyclopropylacetate (MCPA), rat liver mitochondria oxidized palmitoyl–carnitine only as far as butyrate and at a decreased rate. Although pent-4-enoic acid (pent-4-enoate) inhibited the rate of β-oxidation of palmitoyl–carnitine by mitochondria, the oxygen uptake was consistent with the complete oxidation of the substrate. 2. The inhibition of β-oxidation by pent-4-enoate was partially reversed by very high concentrations of L-carnitine. Conditions were also defined for the sustained oxidation of pent-4-enoate by mitochondria. 3. Pent-4-enoate inhibited pyruvate oxidation, but only at concentrations much higher than those needed to inhibit β-oxidation. MCPA had no effect on either pyruvate or 2-oxoglutarate oxidation in mitochondria. 4. Soluble extracts of rat or ox liver mitochondria completely oxidized pent-4-enoyl–CoA and the oxidation of butyryl–CoA added subsequently was unaffected. Incubation of soluble extracts with pent-4-enoyl–CoA in the absence of cofactors caused inhibition of acetoacetyl–CoA thiolase activity. However, this enzyme was not inhibited in intact mitochondria by pent-4-enoate. 5. MCPA specifically inhibited butyryl–CoA dehydrogenase in both intact mitochondria and in soluble extracts supplemented with ATP and CoASH. 6. Both MCPP and MCPA (1 mM) caused a rapid decrease in CoASH concentrations in mitochondria; acetyl–CoA concentrations were unaffected. Concentrations of pent-4-enoate (20 μM) sufficient to inhibit β-oxidation caused only a slight decrease in CoASH whereas higher concentrations (0.1–1.0 mM) caused a more extensive depletion of CoASH. However, evidence is presented to suggest that CoASH depletion is not the mechanism by which these compounds inhibit β-oxidation. 7. Pent-4-enoate and MCPA were substrates for butyryl–CoA synthetase. K_m and V_{max} values for several unusual, straight and branched chain fatty acids were determined. 8. Some short-chain acyl–CoA esters were substrates for an acyl–CoA hydrolase located in the mitochondrial matrix. 9. Some short-chain acyl–CoA esters competitively inhibited the activation of pyruvate carboxylase by acetyl–CoA. 10. The possible mechanisms by which hypoglycin and pent-4-enoate cause inhibition of β-oxidation and hypoglycaemia *in vivo* are discussed.

Ingestion of hypoglycin (2-amino-3-methylenecyclopropylpropionic acid), the toxic hypoglycaemic principle of the unripe arillus of the Jamaican ackee fruit, *Blighia sapida*, causes widespread disturbances of carbohydrate and lipid metabolism [1, 2]. Hypoglycin is converted *in vivo* by transamination to methylenecyclopropylpyruvate (MCPP), which is then oxidatively decarboxylated to the CoA-ester of methylenecyclopropylacetate (MCPA) [3]. Pent-4-enoic acid (pent-4-enoate) is the structurally simplest analogue of MCPA which possesses hypoglycaemic activity [4] and this compound has been extensively studied because it is commercially available and was often assumed to have a similar mechanism of action to MCPA [5]. Pent-4-enoate is first converted to its CoA-ester which then enters the β-oxidation sequence and is metabolized to acetyl–CoA and acryloyl–CoA [6, 7].

It has been proposed that these compounds cause hypoglycaemia by inhibiting gluconeogenesis *in vivo* [8], and we have recently provided direct evidence for this mechanism by a kinetic study using [2-^{3}H, U-^{14}C]glucose in hypoglycin-treated rats [9, 10]. MCPA–CoA interferes with β-oxidation by inactivating butyryl–CoA dehydrogenase (EC 1.3.2.1) so that even-chain length, saturated fatty acids are only shortened as far as butyrate, and probably at a decreased rate [11]. By contrast, the mechanism of the more powerful inhibition of β-oxidation by pent-4-enoate is uncertain although there is little doubt that it is due to a reversible inhibition of an enzyme or enzymes concerned with β-oxidation [2]. We do not accept the alternative view that metabolites of these hypoglycaemic compounds simply impair metabolism by sequestering cellular CoASH as non-metabolizable

Present addresses:

* Department of Chemistry and Biochemistry, Liverpool Polytechnic, Byron Street, Liverpool, L3 3AF, UK.

†Institute for Medical Biochemistry, University of Oslo, Songsvannsveien 9, Oslo 3, Norway.

Abbreviations—MCPP, methylenecyclopropylpyruvate; MCPA, methylenecyclopropylacetate; –CoA, coenzyme A (esterfied form); CoASH, coenzyme A (free form); HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; MOPS, 3-(*N*-morpholino)-propanesulphonic acid; EGTA, ethanedioxybis-(ethylamine)-tetra-acetate; EDTA, ethylenediamine-tetra-acetate; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid).

esters [5]. Further, Tanaka has reported that hypoglycin, but not pent-4-enoate, causes isovaleric and 2-methylbutyric acidaemias by interfering with leucine and isoleucine metabolism [12, 13], and we have since shown that MCPA inactivates isovaleryl–CoA dehydrogenase [14]. It is apparent that complex metabolic disturbances follow these primary inhibitions of some acyl–CoA dehydrogenases.

Although the toxicology of pent-4-enoate has been extensively investigated many aspects of its biochemistry are still uncertain and relatively little direct work has been done on the effects of hypoglycin metabolites on tissue metabolism. This paper describes some further studies on the effects of hypoglycin and its metabolites, and of pent-4-enoate, on CoA-requiring reactions in isolated mitochondria. The following paper describes some of their effects *in vivo* in relation to changes in the activities of tissue enzymes [15]. Preliminary accounts of some of this work have already appeared [16–19].

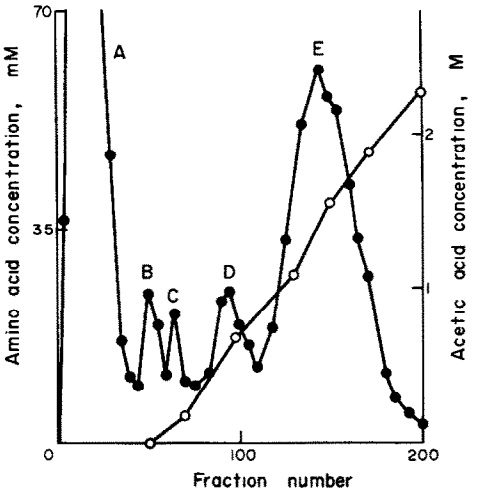

Fig. 1. Purification of hypoglycin by ion exchange chromatography. Details of the column are given in the Methods section. The amino acid concentration of every 5th fraction (●) was determined by the method of Rosen [24] using leucine as standard and the acetic acid concentration of every 20th fraction (○) was determined by titrating 0.2 ml against 1 M NaOH in a Radiometer model 25 pH meter. Amino acids were identified by thin layer chromatography on Eastman Chromogram Sheets No. 6061 (silica gel) developed with n-propanol:water (7:3) and detected with 0.1% (w/v) ninhydrin in ethanol. Peak A contained neutral amino acids, peaks B and C were unidentified, peak D was glutamate and peak E contained a mixture of aspartate and hypoglycin B.

MATERIALS AND METHODS

Animals. Albino, male Wistar rats (250–300 g) and albino, male Balb c mice (25 g), maintained on a standard laboratory diet, were used throughout.

Chemicals. The sources of most chemicals used are given elsewhere [6, 7] except that CoASH, protamine sulphate and phosphotransacetylase (EC 2.3.1.8) were obtained from Sigma Chemical Co. (London) Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K. NADH and NAD^+ were from Boehringer Corp. (London), London W5 2TZ, U.K. HEPES and MOPS were from Hopkins and Williams, Chadwell Heath, Essex, U.K. and all Sephadex and Sepharose gels were from Pharmacia, Upsala, Sweden. All fatty acids and their corresponding acid chlorides and anhydrides were obtained from Fluka A.-G., Buchs, Switzerland except that isovaleryl anhydride was from K & K Laboratories Inc., New York, U.S.A.

Ackee seeds were obtained through the courtesy of the Tropical Products Institute, Chancery Lane, London and Dr. K. E. Magnus, Scientific Research Council, Jamaica.

Preparation of hypoglycin. Hypoglycin (hypoglycin A) occurs in both the unripe arillus and the seed of the ackee fruit whereas hypoglycin B (the γ-glutamyl peptide of hypoglycin) occurs solely in the seed [20]. Hypoglycin can only be chromatographically separated from leucine with great difficulty [21, 22]. However, Fowden [20] and Kean [23] have obtained leucine-free hypoglycin by isolating the acidic amino acids from an extract of ackee seeds by ion exchange chromatography. Acid hydrolysis of this fraction yields free hypoglycin (derived from hypoglycin B) which, because it is neutral, is now easily separated from contaminating acidic amino acids.

Four kg of ground ackee seeds were extracted 3 times with a total of 12 l of 80% (v/v) ethanol. The combined extracts were left overnight, filtered and then evaporated to dryness *in vacuo* at 50°. The residue was dissolved in 700 ml of 0.1 M HCl, emulsified fats were removed by repeated extraction with $CHCl_3$ and the clarified extract was taken to dryness as before. The residue was dissolved in 500 ml of 0.1 M HCl and applied to a column of Dowex-50 (20–50 U.S. mesh, $[H^+]$ form, 4500 ml bed volume). The column was washed with 10 l. of 0.1 M HCl followed by 10 l. of water. The neutral and acidic amino acids were then eluted with approximately 3 bed volumes of 1 M pyridine. The eluate was evaporated to dryness *in vacuo* at 50°, dissolved in 350 ml of water and adjusted to pH 5.5 with NaOH. This was applied to a column of Dowex-1 (20–50 U.S. mesh, acetate form, bed volume 750 ml) which was eluted with an acetic acid gradient (0–3.0 M, 4 l) and 200 fractions of 20 ml collected. The elution profile of this column is illustrated in Fig. 1. Fractions 1–40, which contained neutral amino acids, were combined and evaporated to dryness at 50° *in vacuo*. Four recrystallizations of the residue from 50% (v/v) ethanol gave 4.7 g of hypoglycin. Fractions 110–200, containing hypoglycin B, were combined, evaporated to dryness and the residue refluxed with 500 ml of 2 M formic acid for 5 hr when approximately 70 per cent of the hypoglycin B was hydrolysed to hypoglycin. The hydrolysate was evaporated to dryness, dissolved in 100 ml of water and adjusted to pH 5.5, and then applied to another column of Dowex-1 and eluted as described earlier. The fractions containing hypoglycin (fractions 1–35) were combined and evaporated to dryness. Pure hypoglycin (1.2 g) was obtained after 4 recrystallizations from 50% (v/v) ethanol.

The purity of the hypoglycin preparations was

determined as described by Fincham [25] after reaction with 1% (v/v) Br_2 in glacial acetic acid. Br_2 reacts with the double bond of hypoglycin, but does not react with leucine, enabling leucine to be resolved from the bromination products of hypoglycin with an amino acid analyzer. By comparing chromatograms before and after bromination, it was found that hypoglycin obtained from the neutral amino acid fraction was 85 per cent pure, the impurities being leucine and isoleucine, and that obtained by hydrolysis of hypoglycin B was greater than 99 per cent pure. Hypoglycin refers to the less pure preparation unless otherwise stated. Experience showed that there was no qualitative differences in the effects of the two preparations.

Preparation of methylenecyclopropylpyruvate (MCPP). MCPP was a kind gift from Dr. E. A. Kean, Department of Biochemistry, University of the West Indies, Jamaica and was prepared by a modification of the method of von Holt [3].

Preparation of methylenecyclopropylacetate (MCPA). Hypoglycin was deaminated with ninhydrin to methylenecyclopropylacetaldehyde which was then oxidized to MCPA by ammoniacal silver nitrate (Tollen's reagent). Pure hypoglycin (4 mmol) and ninhydrin (8 mmol) were mixed in 80 ml of water and adjusted to pH 2.3 with 10% (v/v) H_3PO_4 in a distillation flask. Steam was then passed through the mixture and the distillate containing methylenecyclopropylacetaldehyde collected at 0° until it no longer gave a yellow precipitate with 2,4-dinitrophenylhydrazine (0.1% w/v in 2 M HCl). The aldehyde was oxidized by adding 1.1 times the theoretical amount of Tollen's reagent (8.8 mmol of $AgNO_3$). The mixture was stirred in the dark for 30 min and then filtered. The filtrate was passed through a column of Dowex-50 ($[H^+]$ form) with a capacity of 10 times the expected cation content of the mixture (about 120 m-equiv). The eluate was collected until the pH rose to 6.0; this contained free MCPA. It was isolated as the potassium salt by adding 2 M KOH until the pH was 6.5. The solution was freeze-dried and the light residue recrystallized from hot ethanol to give the potassium salt of MCPA in approximately 80 per cent yield.

Preparation of acyl–carnitine esters. Acyl-L-carnitine esters were prepared and characterized as described previously [6].

Preparation of acyl–CoA esters. Acetyl–CoA, butyryl–CoA, n-pentanoyl–CoA and hexanoyl–CoA were prepared by reaction of CoASH with the corresponding acid anhydride [26]. Pent-4-enoyl–CoA, isovaleryl–CoA, 2-methylbutyryl–CoA, isobutyryl–CoA and acryloyl–CoA were prepared by reaction of CoASH with the mixed anhydride of the corresponding acid and ethyl chloroformate [27]. The concentrations of the acyl-CoA solutions were assayed using carnitine acetyltransferase (EC 2.3.1.7) [28]. However, the branched chain acyl–CoA esters were not effective substrates for carnitine acetyltransferase and the concentrations of these solutions were determined by the hydroxamate method [29]. Acetoacetyl–CoA was prepared by using diketen as described by Weiland and Rueff [30] and assayed with β-hydroxybutyryl–CoA dehydrogenase [7]. It was not possible to prepare 3-hydroxypent-4-enoyl–CoA or 3-oxopent-4-enoyl–CoA.

Preparation of 2-oxoglutarate dehydrogenase (EC 1.2.4.2). 2-Oxoglutarate dehydrogenase was prepared to the stage of precipitation from a pig heart extract by 0.02% (w/v) protamine sulphate as described by Hirashima *et al.* [31]. The precipitate was resuspended in 50 mM potassium phosphate, pH 7.0, dialysed overnight against this buffer and the resultant solution applied to a column (3 × 50 cm) of Sepharose 6B and eluted with the same buffer. The peak of activity was pooled, concentrated and stored at −20° in several batches in 20% (v/v) glycerol. The specific activity assayed at 20° by the method of Massey [32], was 0.1 units/mg of protein.

Preparation of pyruvate carboxylase (EC 6.4.1.1). Pyruvate carboxylase was prepared to the stage of precipitation from a pig liver mitochondrial extract by 20% (w/v) $(NH_4)_2SO_4$ as described by Warren and Tipton [33]. The precipitate was resuspended in 100 mM triethanolamine hydrochloride–KOH buffer, pH 8.0, dialysed overnight against this buffer and the resultant solution applied to a column (3 cm × 50 cm) of Sephadex G-200 and eluted with this buffer. The peak of activity was pooled, concentrated and stored at −20° in 25% (v/v) glycerol. The specific activity, assayed at 20° and pH 7.4 in the direction of malate formation [33], was 0.1 units/mg of protein.

Preparation of butyryl–CoA (medium chain) synthetase (EC 6.2.1.2). Butyryl–CoA synthetase was prepared from acetone-dried rat liver mitochondria as described by Osmundsen and Park [34]. It had a specific activity of 20 units/mg of protein at 20° with sorbate as substrate [35].

Preparation of acyl–CoA hydrolase (EC 3.1.2.–). Crude preparations of acyl–CoA hydrolase were prepared by extracting acetone-dried ox liver mitochondria with 10 mM potassium phosphate, pH 7.4, centrifuging at 130,000 *g* for 60 min and passing the supernatant through a column (3 cm × 50 cm) of Sephadex G-25 pre-equilibrated with 10 mM potassium phosphate. Activities were measured by following the release of −SH groups from various acyl–CoA esters with 0.5 mM aldrithiol-4 at pH 7.2 and 20° [11].

Other enzyme assays. The oxidation of acyl–CoA esters by butyryl–CoA (short chain) dehydrogenase (EC 1.3.2.1) or by palmitoyl–CoA (long chain) dehydrogenase (EC 1.3.2.2) was assayed at 20° by following the reduction of cytochrome *c* at 550 nm in the presence of 30 μM acyl–CoA and 0.5 mM phenazine methosulphate [7].

Acetoacetyl–CoA thiolase (EC 2.3.1.9) was assayed at 20° by measuring the decrease in optical density at 303 nm due to the thiolysis of 50 μM acetoacetyl–CoA in the presence of 100 mM Tris–HCl, pH 8.1, 40 μM CoASH and 4 mM $MgCl_2$ [7].

β-Hydroxybutyryl–CoA dehydrogenase (EC 1.1.1.35) was assayed at 20° by recording the decrease in optical density at 340 nm in 50 mM Tris–HCl, pH 7.5, 50 μM acetoacetyl–CoA and 50 μM NADH, final volume 1.5 ml [7].

All enzyme assays contained 15–200 μg of enzyme protein per ml of reaction mixture.

Preparation of mitochondria. Rat or ox liver mitochondria were isolated in 0.3 M mannitol, 5 mM HEPES, 0.1 mM EGTA, pH 7.2 [36] and were used immediately or dried with acetone [37].

Measurement of oxygen uptake by mitochondria.

Oxygen uptake by suspensions of mitochondria was recorded polarographically at 30° in a final volume of 3.0 ml of 120 mM KCl, 5 mM HEPES, 2.5 mM phosphate, 1 mM EDTA adjusted to pH 7.2 with KOH. When measuring rates of acyl–carnitine oxidation 10 mM malonate was added so that acyl-groups were quantitatively converted to acetoacetate and the rate of oxygen uptake was proportional to the flux through β-oxidation [6]. Respiration in the absence of a phosphate acceptor is termed state 4, and in the presence of 1 mM ADP or uncoupler (20 μM 2,4-dinitrophenol) is termed state 3 or state 3u respectively [38].

Measurement of β-oxidation in mitochondrial extracts. Acetone-dried rat or ox liver mitochondria (2 g) were extracted with 10 ml of 10 mM potassium phosphate, pH 7.2, and the extract centrifuged at 130,000 *g* for 60 min at 4°. Low molecular weight compounds were removed by passing the supernatant through a column (2.5 × 40 cm) of Sephadex G-25 pre-equilibrated with 10 mM potassium phosphate, pH 7.2, to give a final protein concentration of about 5 mg/ml. β-oxidation of acyl–CoA esters by this extract was followed polarographically essentially as described by Stewart *et al.* [39] at 30° in a final volume of 3.0 ml of 10 mM potassium phosphate, pH 7.2. The medium was supplemented with 0.2 mM CoASH and 0.3 mM NAD^+, and 50 μM phenazine methosulphate and 0.001% (w/v) methylene blue were added as artificial electron carriers to couple β-oxidation to O_2. It was also found necessary to add 125 μg of catalase (EC 1.11.1.6) to decompose H_2O_2 formed by reaction of reduced methylene blue with O_2.

Assay of CoASH and acetyl–CoA in isolated mitochondria. A modified Aminco–Bowman spectrofluorimeter was used to assay CoASH. The excitation light beam was interrupted at 60 Hz by a rotating vane placed between the xenon light source and the excitation monochromator. The output from the emission monochromator was detected by an EMI 9592B photomultiplier tube and, together with the frequency of interruption reference light beam, were monitored on a Tektronix RM 565 dual beam oscilloscope and then fed into a Brookdeal lock-in amplifier, model 9501. The amplifier differentiates between noise and signal and subtracts the former from the latter. The output was displayed on a Servoscribe pen recorder. These modifications allowed a full scale deflection of 1–2 nmol of NADH with a noise level of < 1 per cent f.s.d.

Mitochondria were incubated with stirring at 30° at a final protein concentration of 3–6 mg/ml in 14 ml of 120 mM KCl, 10 mM MOPS, 5 mM $MgCl_2$, 2.5 mM phosphate, 2 mM ADP, 1 mM EDTA, pH 7.2. Samples of 2 ml were taken at appropriate time intervals and added to 1.0 ml of 10% (v/v) $HClO_4$ and prepared for analysis as described by Williamson and Corkey [40]. Free CoASH was assayed at 20° and pH 7.2 in a final volume of 2.0 ml containing 50 mM K_3AsO_4, 50 μM 2-oxoglutarate, 20 μM NAD^+ and 50 μM dithiothreitol [41]. The reaction was started by the addition of 2-oxoglutarate dehydrogenase and the increase in fluorescence at 466 nm recorded. When the reaction was complete (4–5 min), acetyl–CoA was determined in the same cuvette as the CoASH released following the addition of 1 unit of phosphotransacetylase.

Determination of protein. Protein was determined by the method of Lowry *et al.* [42] using dried bovine serum albumin as standard. Suspensions of mitochondria were first clarified by adding 1% (v/v) Triton X-100.

RESULTS AND DISCUSSION

Pent-4-enoate must first be converted to pent-4-enoyl–CoA to cause inhibition of β-oxidation [6]. MCPA–CoA is the inhibitory species derived from free MCPA or hypoglycin [11]. Many features of the effects of pent-4-enoate, pent-4-enoyl–carnitine and MCPA on CoA-dependent reactions in mitochondria have already been reported from this laboratory [6–8, 11, 36, 43]. A comprehensive understanding of the molecular events leading to inhibition of β-oxidation and gluconeogenesis is still, however, lacking.

Effects of pent-4-enoate on β-oxidation in intact mitochondria

Previously we reported that low concentrations (10–100 μM) of pent-4-enoyl–carnitine powerfully inhibited both its own oxidation and the β-oxidation of acyl–carnitine esters of all chain lengths by rat liver mitochondria [6]. This is in marked contrast to the sustained oxidation of quite high concentrations (up to, but not greater than, 1.7 mM) of pent-4-enoate by perfused rat livers [44] and to the high concentrations (1 mM) needed to inhibit the evolution of $^{14}CO_2$ from [1-^{14}C]palmitate in tissue homogenates [45]. It has been reported that the addition of L-carnitine and CoASH reversed the inhibition of β-oxidation by pent-4-enoate in homogenates [45, 46]; however we earlier failed to reverse the inhibition by 50 μM pent-4-enoate in isolated mitochondria by the addition of 2 mM L-carnitine and 0.2 mM CoASH [6]. Some further experiments were therefore done with pent-4-enoate to try to resolve these discrepancies.

It was confirmed that 10 μM pent-4-enoate inhibited the state 3 rate of oxidation of 10 μM palmitoyl–carnitine by 60 per cent (Fig. 2). The oxygen uptake was consistent with the complete oxidation of the substrate to acetoacetate (Fig. 2), in contrast to the incomplete oxidation of palmitoyl–carnitine to butyrate in the presence of MCPA [11].

It was possible to reverse partially this inhibition of palmitoyl–carnitine oxidation by the addition of high concentrations of L-carnitine; Fig. 3a shows that in the presence of 10 μM pent-4-enoate and 10 mM L-carnitine, palmitoyl–carnitine oxidation was inhibited by only 18 per cent. (L-carnitine alone had no significant effect on the control rate of palmitoyl–carnitine oxidation). The higher concentration of L-carnitine and the lower concentration of pent-4-enoate used in this study presumably facilitates the removal of inhibitory acyl–CoA species from the matrix at a greater rate than they can be formed.

It was also possible to define conditions for the sustained oxidation of low concentrations of pent-4-enoate (< 300 μM) in the presence of a high concentration of L-carnitine (10 mM) (Fig. 3b). The oxida-

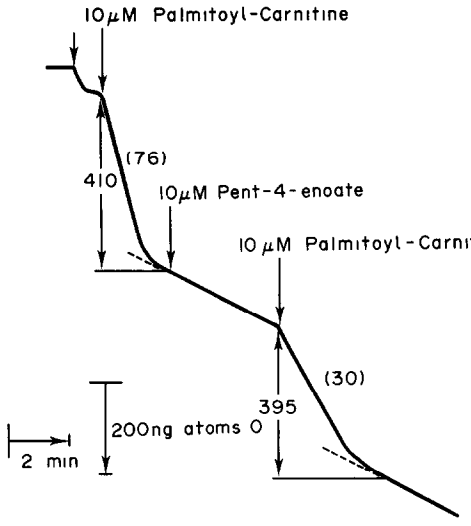

Fig. 2. The state 3 oxidation of palmitoyl-carnitine in the presence of 10 μM pent-4-enoate. Rat liver mitochondria (about 5 mg of protein per incubation) were added where indicated (by unlabelled arrow) followed by 10 μM palmitoyl-carnitine, 10 μM pent-4-enoate and finally 10 μM palmitoyl-carnitine. The amount of oxygen consumed (ng atoms O) is indicated by the vertical arrows and the rates of oxygen uptake (ng atoms O/min/mg of protein) are given in parentheses. It was assumed that endogeneous respiration was suppressed during the oxidation of palmitoyl-carnitine [11].

tion of 12 μM palmitoyl-carnitine added subsequently was completely inhibited (Fig. 3b) showing that the inhibition of palmitoyl-carnitine oxidation can be dissociated from the inhibition of pent-4-enoate oxidation. However, even with these conditions the oxidation of concentrations of pent-4-enoate greater than 300 μM was self inhibitory.

Effects of pent-4-enoate and MCPA on the oxidation of pyruvate and 2-oxoglutarate

Pent-4-enoate inhibits the oxidation of pyruvate and 2-oxoglutarate by rat liver mitochondria [36, 47]. However, this effect is non-specific in that the non-hypoglycaemic, structurally related fatty acids pent-2-enoate, cyclopropanecarboxylate and cyclobutane-carboxylate similarly inhibit the oxidation of these substrates [36, 47]. Indeed, the oxidation of pyruvate is less sensitive to inhibition by pent-4-enolate than is β-oxidation, and under some conditions, β-oxidation can be inhibited in the absence of significant inhibition of pyruvate oxidation [6]. For these reasons we have stressed the importance of using chemically similar but non-hypoglycaemic fatty acids as controls in this type of investigation [2, 6].

MCPA had no effect on either pyruvate or 2-oxoglutarate oxidation by rat liver mitochondria at concentrations (10–100 μM) that caused maximal inhibition of palmitoyl-carnitine oxidation. von Holt *et al.* have also shown that 60 μM MCPA did not inhibit the release of $^{14}CO_2$ from [2-^{14}C]pyruvate by rat liver mitochondria [48].

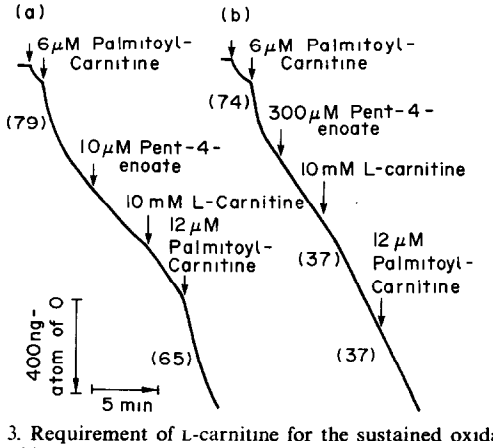

Fig. 3. Requirement of L-carnitine for the sustained oxidation of low concentrations of pent-4-enoate. Rat liver mitochondria (about 5 mg of protein per incubation) were added where indicated (by unlabelled arrows); palmitoyl-carnitine, L-carnitine and (a) 10 μM pent-4-enoate, or, (b) 300 μM pent-4-enoate were then added as shown. The rates of oxygen uptake (ng atoms O/min/mg of protein) are given in parentheses.

Effects of pent-4-enoate and MCPA on β-oxidation in soluble mitochondrial extracts

Soluble extracts of rat or ox liver mitochondria rapidly and completely oxidized 0.5 mM butyryl-CoA and 0.5 mM hexanoyl-CoA to acetyl-CoA at approximately 270 ng atoms O/min/mg of protein. In contrast with intact mitochondria [6], these extracts also completely oxidized 0.1 mM pent-4-enoyl-CoA, and 0.1 mM n-pentanoyl-CoA at about one-third of the rate of the oxidation of butyryl-CoA, presumably to acetyl-CoA and acryloyl-CoA or propionyl-CoA respectively. There was no impairment of the rate of oxidation of butyryl-CoA, or of hexanoyl-CoA, added after the completion of the oxidation of 0.1 mM pent-4-enoyl-CoA.

When 0.1 mM pent-4-enoyl-CoA was incubated with mitochondrial extracts in the presence of electron acceptors but in the absence of cofactors, the extent of oxygen uptake was consistent with its conversion to pent-2,4-dienoyl-CoA by butyryl-CoA dehydrogenase. The extracts were then passed again through a column of Sephadex G-25 and acetoacetyl-CoA thiolase, butyryl-CoA dehydrogenase and β-hydroxybutyryl-CoA dehydrogenase activities were assayed in the eluate. A similar incubation with 0.1 mM n-pentanoyl-CoA, which was quantitatively oxidized to pent-2-enoyl-CoA, was done as control. In these incubations acetoacetyl-CoA thiolase was inhibited by 60–70 per cent in extracts of ox liver mitochondria and by 30 per cent in extracts of rat liver mitochondria that had been incubated with pent-4-enoyl-CoA (Table 1). There was no inhibition of butyryl-CoA dehydrogenase or β-hydroxybutyryl-CoA dehydrogenase (Table 1). Similar results were obtained when 0.1 mM pent-4-enoate or n-pentanoate and 10 mM MgATP were used to generate the CoA-esters in the extracts. Any inhibition of acetoacetyl-CoA thiolase would be distal in the β-oxidation sequence to the dehydrogenases coupled to O_2 with artificial electron acceptors, and would therefore not cause a decreased rate of O_2 uptake. However, the absence of inhibition of the oxidation of hexanoyl-CoA indicates that, in this system, the general 3-oxoacyl-CoA thiolase (EC 2.3.1.16) was not inhibited sufficiently to be rate limiting in β-oxidation.

Table 1. Effect of pent-4-enoyl–CoA on the activities of acetoacetyl–CoA thiolase and butyryl–CoA dehydrogenase in mitochondrial extracts

Mitochondrial extract	Addition	Specific activity (nmol/min/mg of protein) Acetoacetyl–CoA thiolase	Butyryl–CoA dehydrogenase	β-Hydroxybutyryl–CoA dehydrogenase
Ox	n-Pentanoyl–CoA	203 ± 10	32 ± 2	1020 ± 56
	Pent-4-enoyl–CoA	87 ± 7	35 ± 2	1220 ± 44
Rat	n-Pentanoyl–CoA	210 ± 12	62 ± 1	160 ± 2
	Pent-4-enoyl–CoA	150 ± 5	61 ± 4	160 ± 9

Soluble extracts of ox or rat liver mitochondria were incubated with 0.1 mM pent-4-enoyl–CoA, or, in the case of controls, 0.1 mM n-pentanoyl–CoA, as described in the text. The extracts were then passed through a column of Sephadex G-25 and assayed for enzyme activities. β-Hydroxybutyryl–CoA dehydrogenase (which was not inhibited) was assayed as an internal standard. Values are means of two separate experiments ± S.E.M.

Incubation of rat or ox liver mitochondrial extracts for 5 min with 60 μM MCPA caused complete inactivation of butyryl–CoA dehydrogenase whilst acetoacetyl–CoA thiolase was not inhibited; both ATP and CoASH were necessary for the development of this inhibition and addition of MCPA did not cause any O_2 uptake. Inhibition of butryl–CoA dehydrogenase was not reversed by prolonged dialysis of the inhibited extract, nor by removing low molecular weight compounds by passing the inhibited extract through a column of Sephadex G-25. These results, and those of Osmundsen and Sherratt using purified butyryl–CoA dehydrogenase and butyryl–CoA synthetase [11], indicate that the inhibitory species is MCPA–CoA and that this binds very tightly or irreversibly to the dehydrogenase. We failed to prepare MCPA–CoA suggesting that this ester is unstable. However, Kean subsequently succeeded and showed that it inhibited partially purified rabbit butyryl–CoA dehydrogenase with an apparent K_i of 20 μM [49]. We have also shown that incubation of rat liver mitochondria with 1 mM MCPP or with 1 mM MCPA caused a 50 per cent decrease in the state 3 rate of oxidation of palmitoyl–carnitine and virtually complete inhibition of butyryl–CoA dehydrogenase in extracts of these mitochondria [16], although there was no inhibition of acetoacetyl–CoA thiolase.

When intact mitochondria were incubated with 1 mM pent-4-enoate there was no impairment of butyryl–CoA dehydrogenase, nor of acetoacetyl–CoA thiolase, measured in extracts of these mitochondria when compared with mitochondria incubated with 1 mM n-pentanoate (Table 2). This difference in the inhibition of acetoacetyl–CoA thiolase in intact mitochondria and in mitochondrial extracts was unexpected. However, some structural organisation of the enzymes of β-oxidation may exist within the matrix [50], and this may afford some protection against inactivation of an enzyme by metabolites that are not analogues of its substrate.

It may be concluded that an *irreversible* inactivation of acetoacetyl–CoA thiolase is not involved in the inhibition of β-oxidation in intact mitochondria by pent-4-enoate and that MCPA–CoA specifically inhibits β-oxidation by inactivating butyryl–CoA dehydrogenase.

Localization of isooxocaproate dehydrogenase in rat liver mitochondria

Hypoglycin is transaminated by reaction with 2-oxoglutarate to give MCPP, and, since hypoglycin

Table 2. Effect of pent-4-enoate on the activities of acetoacetyl–CoA thiolase and butyryl–CoA dehydrogenase in intact rat liver mitochondria

Addition	Specific activity (nmol/min/mg of protein) Acetoacetyl–CoA thiolase	Butyryl–CoA dehydrogenase	β-Hydroxybutyryl–CoA dehydrogenase
n-Pentanoate	100 ± 4	89 ± 10	510 ± 11
Pent-4-enoate	107 ± 7	100 ± 7	540 ± 20

Mitochondria were incubated for 10 min at a final protein concentration of 5 mg/ml with either 1 mM pent-4-enoate, or, in the case of controls, 1 mM n-pentanoate, in 120 mM KCl, 10 mM malonate, 10 mM HEPES, 3 mM phosphate, 2 mM EDTA, 1 mM ADP, pH 7.2 in 3 ml at 30°. The suspensions were then solubilized by the addition of 1% (v/v) Triton X-100 and the supernatants resulting after centrifugation at 200,000 *g* for 30 min assayed for enzyme activities. β-Hydroxybutyryl–CoA dehydrogenase (which was not inhibited) was assayed as an internal control. Similar 5 mg/ml with either 1 mM pent-4-enoate, or, in the case of controls, 1 mM n-pentano- with acetone and the enzymes assayed in their soluble extracts. Values are means of two separate experiments ± S.E.M.

competitively inhibits leucine transamination in rat liver slices [51], it is likely that the transamination of hypoglycin is also catalysed by leucine aminotransferase (EC 2.6.1.6), which is located in the cell cytosol [52]. MCPP is then presumably oxidatively decarboxylated to MCPA–CoA by isooxocaproate dehydrogenase, the enzyme which converts isooxocaproate (derived from leucine) to isovaleryl–CoA. It has been reported that isooxocaproate dehydrogenase is located on the outer face of the inner membrane in ox liver mitochondria [53]. If this were true for rat liver mitochondria, MCPA–CoA would have to be transferred into the mitochondrial matrix by the action of the carnitine acyltransferases associated with the inner mitochondrial membrane in order to inhibit β-oxidation. However, we have found that MCPP and MCPA inhibit β-oxidation in the absence of either CoASH or L-carnitine [11, 16]. Further, there is evidence that branched chain acyl–CoA esters, and by implication MCPA–CoA, are very poor substrates for the carnitine acyltransferases [54] and it has never been determined whether MCPA groups can be transferred to L-carnitine. Bremer has recently shown that the oxidation of branched chain ketoacids involves intramitochondrial NAD^+ and CoASH [55], and therefore it seems likely that isooxocaproate dehydrogenase is located in the matrix of rat liver mitochondria.

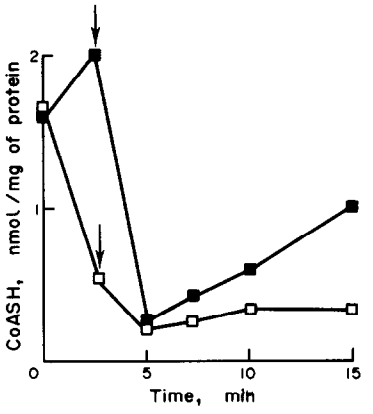

Fig. 4. Effect of MCPP on CoASH concentrations in rat liver mitochondria. Mitochondria were incubated in state 3 conditions as described in the Methods section. 1 mM MCPP (□), or, in the case of the control, an equivalent volume of incubation medium (■) was added at zero time. 0.1 mM Palmitoyl–carnitine was added where indicated (by unlabelled arrows). The incubations were sampled immediately before and at 3, 5, 7, 10 and 15 min after the addition of MCPP and assayed for CoASH as described in the Methods section.

Effects of MCPP, MCPA and pent-4-enoate on CoASH and acetyl–CoA concentrations in rat liver mitochondria

Incubation of mitochondria with 1 mM MCPP in state 3 produced a rapid and extensive decrease in CoASH concentrations (Fig. 4). The addition of 0.1 mM palmitoyl–carnitine produced an equally rapid and extensive decrease in CoASH concentrations (Fig. 4). However, CoASH concentrations slowly increased in these mitochondria (Fig. 4); presumably this reflects the rapid and complete oxidation of palmitoyl–carnitine compared with its slow and incomplete oxidation in the presence of MCPA. In both cases CoASH concentrations did not fall below 0.3 nmol/mg of protein (Fig. 4). Acetyl–CoA concentrations remained constant at 0.4–0.6 nmol/mg of protein both in the presence and absence of MCPP (not shown).

Incubation with 1 mM MCPA produced a similar decrease in intramitochondrial CoASH concentrations (Fig. 5). However, this was less rapid and extensive than that produced by MCPP (Fig. 4). This is probably because MCPP is oxidatively decarboxylated to MCPA–CoA by isooxocaproate dehydrogenase whereas MCPA has to be converted to MCPA–CoA by butyryl–CoA synthetase which has high K_m values for its substrates (see later). Again acetyl–CoA concentrations were constant in both control and MCPA-treated mitochondria (not shown).

Mitochondria incubated with 20 μM pent-4-enoate for 3 min had their ability to oxidize 10 μM palmitoyl–carnitine decreased by 50 per cent whilst the rate of oxidation of 10 mM pyruvate was not inhibited. Higher concentrations of pent-4-enoate (> 0.1 mM) inhibited both palmitoyl–carnitine and pyruvate oxidation. Mitochondria incubated with 20 μM pent-4-enoate contained 1.3 nmol CoASH/mg of protein compared to 1.7 nmol CoASH/mg of protein in its absence. By contrast, mitochondria incubated under the same conditions with 1 mM pent-4-enoate contained only 0.2 nmol CoASH/mg of protein. Holland and Sherratt also found that mitochondria similarly incubated with 0.1 mM pent-4-enoate contained 0.1–0.2 nmol CoASH/mg of protein [6].

The control values for the intramitochondrial concentrations of CoASH and acetyl–CoA are in good agreement with other values in the literature [41, 56, 57].

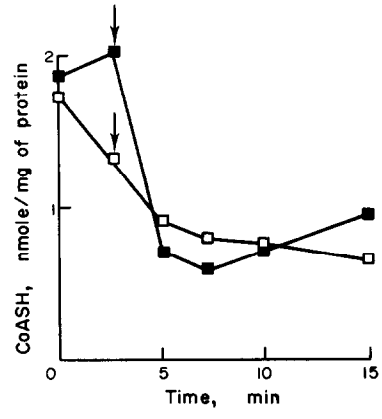

Fig. 5. Effect of MCPA on CoASH concentrations in rat liver mitochondria. 1 mM MCPA (□), or, in the case of the control, an equivalent volume of incubation medium (■) was added at zero time. 0.1 mM palmitoyl–carnitine was added where indicated (by unlabelled arrows). Other details are given in the legend to Fig. 4.

CoASH is essentially confined to the matrix in isolated mitochondria with a total concentration of all CoA-species of 3–7 mM, using values of 0.5–1.0 μl of matrix water/mg of protein as determined in this laboratory [58]. Even mitochondria incubated with 1 mM pent-4-enoate therefore have CoASH concentrations of 200–400 μM. Unless it is assumed that most of this CoASH is protein bound, this concentration is several times higher than the reported K_m values for CoASH of the various enzymes involved in β-oxidation. This, together with the fact that inhibition of β-oxidation can be dissociated from that of pyruvate oxidation, indicates that sequestration of CoASH cannot be the cause of inhibition of β-oxidation by either pent-4-enoate or by MCPA. Indeed, uninhibited β-oxidation is known to suppress pyruvate oxidation [59].

Effects of metabolites of hypoglycin and pent-4-enoate on some isolated enzymes

Butyryl–CoA synthetase. Butyryl–CoA synthetase is located in the mitochondrial matrix [60] and activates a range of medium chain fatty acids [61]. Table 3 shows that the rat liver enzyme activates a range of unusual, branched and straight chain fatty acids with high K_m values. We have also presented indirect evidence that MCPA is a substrate (see earlier and ref. [11]). With the assay conditions used there was a high apparent K_m (6.3 mM) for pent-4-enoate although the V_{max} values were similar for butyrate and pent-4-enoate (Table 3); how far these values apply to conditions in the matrix is unknown. Organic acids will tend to be concentrated in the matrix since this becomes more alkaline during respiration [63], and this may partly compensate for the high K_m values of some of them for the synthetase.

Although the K_m for cyclopropanecarboxylate was higher than that for pent-4-enoate and the V_{max} was 85 per cent lower (Table 3), Holland and Sherratt have shown that it rapidly acylates intramitochondrial CoASH [6]. However, these workers did not detect acylation of CoASH by short-chain acyl groups when mitochondria were incubated with 1.0 mM acrylate [6] although it is a substrate for the synthetase (Table 3).

Table 3. Kinetic constants of rat liver butyryl–CoA synthetase for some unusual, medium chain fatty acids

Substrate	Apparent K_m (mM)	Apparent V_{max} (μmol/min/mg or protein)
Butyrate	0.5 ± 0.04	1.4 ± 0.2
Pent-4-enoate	6.3 ± 0.1	1.4 ± 0.1
Acrylate	10.0 ± 0.4	1.4 ± 0.3
Cyclopropanecarboxylate	10.0 ± 0.6	0.22 ± 0.06
Cyclobutanecarboxylate	20.0 ± 1.0	0.22 ± 0.08

Butyryl–CoA synthetase was assayed essentially as described by Bar-Tana *et al.* [62] in the presence of saturating concentrations of CoASH (0.1 mM) and MgATP (10 mM) in a final volume of 0.9 ml containing 50 mM Tris–HCl, pH 8.2, 20 mM $KHCO_3$ and varying concentrations of substrate. The mixture was incubated at 30° for 10 min and then 1.0 ml of 0.2 M NaH_2PO_4, pH 8.2 and 0.1 ml of 10 mM DTNB were added. Reaction mixtures without fatty acid substrates served as controls. Reaction velocities were calculated (assuming a molar extinction coefficient of $13.6 \times 10^4\ cm^{-1}$) from the decrease in colour due to disappearance of CoASH. Values for K_m and V_{max} were determined from classical Lineweaver–Burk double reciprocal plots. Values are means of 2 separate experiments ±S.E.M.

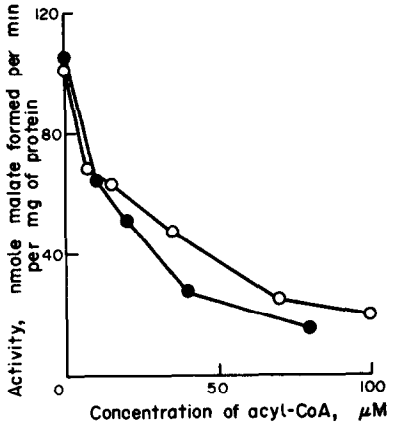

Fig. 6. Inhibition of pig liver pyruvate carboxylase by butyryl–CoA and pent-4-enoyl–CoA. The enzyme was assayed at pH 7.4 and 20° as described by Warren and Tipton [31]. The concentration of acetyl–CoA in the assay system was 16 μM and butyryl–CoA (●) or pent-4-enoyl–CoA (○) were added at the final concentration shown.

Acyl–CoA hydrolase. The crude enzyme preparation deacylated butyryl–CoA, hexanoyl–CoA and acryloyl–CoA with apparent K_m values of 2 mM, 0.5 mM and 70 μM respectively. V_{max} values for butyryl–CoA and hexanoyl–CoA were 10 and 15 nmol/min/mg of protein respectively. These esters may be split by acetyl–CoA hydrolase (EC 3.1.2.1) which has been found in the mitochondrial matrix and partly characterized [64]. However, we could not detect hydrolysis of pent-4-enoyl–CoA at concentrations of up to 2 mM *in vitro*.

Pyruvate carboxylase. Acetyl–CoA is an obligatory activator for this key gluconeogenic enzyme [65]. We have confirmed that the pig liver enzyme requires acetyl–CoA for activity with a K_a value of 9.8 μM. With the same assay conditions Warren and Tipton found a value of 4.8 μM [33]. Both butyryl–CoA (K_i, 15 μM) and pent-4-enoyl–CoA (K_i, 19 μM) competitively inhibited this activation (Fig. 6). Isovaleryl–CoA and 2-methylbutyryl–CoA also inhibited this activation (by approximately 50 per cent at 10 μM) although no K_i values were determined.

Fate of medium chain fatty acids in the mitochondrial matrix

Before considering the possible mechanisms by which metabolites of hypoglycin and pent-4-enoate inhibit β-oxidation and gluconeogenesis, it is pertinent to consider the fate of medium chain fatty acids in the mitochondrial matrix. Medium chain fatty acids are converted to their CoA-esters by butyryl–CoA synthetase; this reaction is probably irreversible because of the association of this enzyme with pyrophosphatase (EC 3.6.1.1) and is a major source of

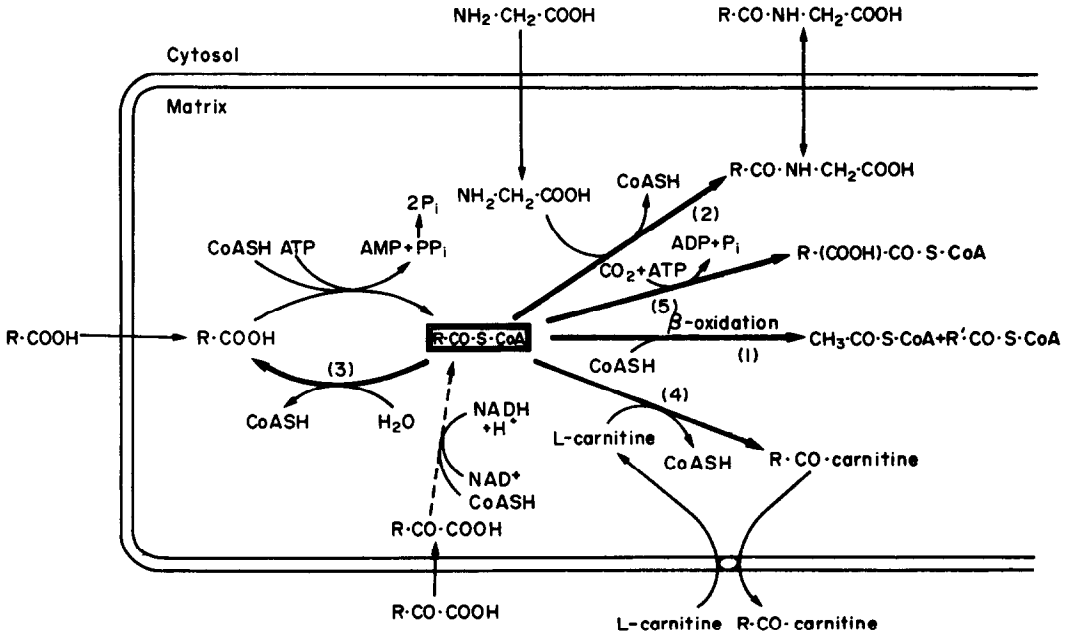

Fig. 7. Some competing reactions for acyl–CoA esters in the mitochondrial matrix. The numbers refer to reactions quoted in the text.

acyl–CoA species. Branched-chain acyl–CoA species may also arise from the oxidative decarboxylation of 2-oxo acids. Acyl–CoA esters formed in the matrix may then undergo several possible reactions and those which are relevant to our arguments are shown in Fig. 7. (1) Acyl–CoA esters with the appropriate structure may undergo some or all of the reactions of β-oxidation. The β-oxidation system has a very high affinity for its substrates and it is difficult to detect intermediates [50]. Some unusual acyl–CoA esters may, however, be generated by β-oxidation which cannot be metabolized to acetyl–CoA, and these may then undergo some of the reactions listed below. (2) Many unusual acyl–CoA esters react irreversibly with glycine to form conjugates. This reaction is catalysed by glycine *N*-acylase (EC 2.3.1.13) which is located in the matrix [66]. The enzyme has low K_m values for many acyl–CoA esters (0.01–0.20 mM) but a high K_m value for glycine (3–15 mM) [67]. (3) They may be hydrolysed back to the free acid and CoASH. Such enzymes appear to have very high K_m's for their substrates and act primarily to oppose complete acylation of mitochondrial CoASH. (4) Many acyl–CoA esters react reversibly with L-carnitine enabling transfer of acyl-groups out of the matrix; different esters react at widely differing rates [6, 48]. Further, some acyl–carnitine esters formed may leave the matrix and then be hydrolysed by an acyl–carnitine hydrolase associated with the outer mitochondrial membrane [69]. (5) Finally, some acyl–CoA esters, particularly propionyl–CoA and branched-chain acyl –CoA esters, may undergo ATP-dependent carboxylation reactions.

The effects of each medium-chain fatty acid on CoA-dependent reactions in the matrix will be different because of competing pathways, different K_m and V_{max} values of each of these enzymes for each fatty acid or its CoA-ester, and because of variations in other factors such as the concentrations of L-carnitine, glycine or CO_2 in the system.

Mechanisms of inhibition of β-oxidation

Metabolites of both pent-4-enoate and hypoglycin inhibit β-oxidation as well as gluconeogenesis in several *in vitro* preparations [6, 7, 11, 16–18, 36, 43–49]. However, it is now clear that these agents have differences in their mechanisms of action [2]. The time course of the hypoglycaemia caused by hypoglycin is much longer than that caused by pent-4-enoate [15]. Further, hypoglycin, but not pent-4-enoate, inhibits the metabolism of some branched chain amino acids [12, 13, 15].

Inhibition of β-oxidation by metabolites of hypoglycin. The present results support the conclusion of Osmundsen and Sherratt that β-oxidation is inhibited as a result of the irreversible inactivation of butyryl–CoA dehydrogenase by MCPA–CoA such that acyl-groups are shortened only as far as butyrate [11]. It is likely that the impaired state 3 rate of partial β-oxidation is then determined by either a feedback inhibition on some other enzyme of β-oxidation by accumulated butyryl–CoA or by the rate of deacylation of butyryl–CoA by acyl–CoA hydrolase. This enzyme has a high apparent K_m for butyryl–CoA (2 mM) and a rapid rate of deacylation requires a large proportion of the total matrix CoASH in the form of butyryl–CoA. The CoASH content of MCPA-inhibited mitochondria suggests that the concentration of butyryl–CoA is of the order of 4 mM when palmitoyl–carnitine oxidation is inhibited by 50 per cent (see Fig. 8).

Deacylation of accumulated butyryl–CoA may also occur by two other reactions (see Fig. 7). However, glycine *N*-acylase has a very high K_m for glycine [67], and, although the reaction of butyryl–CoA with L-carnitine is freely reversible [7], this would only

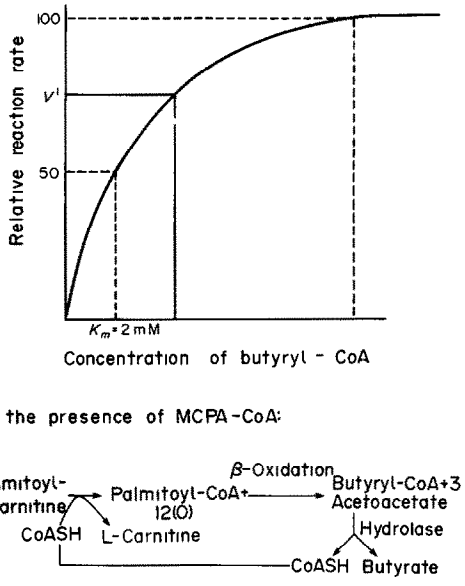

Fig. 8. Relationship of the concentration of butyryl–CoA in the matrix to the rate of partial β-oxidation in the presence of MCPA. When the β-oxidation of palmitoyl–carnitine is inhibited in isolated mitochondria by MCPA the remaining rate is of the order of 40 ng atoms O/min/mg of mitochondrial protein (see refs [11] and [17]). If in steady state conditions the flux through β-oxidation is only limited by the rate of recycling of CoASH by acyl–CoA hydrolase, the rate of hydrolysis of butyryl–CoA will = 40/12 = 3.3 nmol/min/mg of mitochondrial protein. The apparent K_m of acyl–CoA hydrolase for butyryl–CoA was 2 mM and assuming simple hyperbolic kinetics apply to the conditions in the matrix, then a steady state concentration of 4 mM butyryl–CoA would be expected. Alternatively, if the rate of β-oxidation is limited by some other factor, which then determines the rate of generation of butyryl–CoA, the rate of hydrolysis, v', would correspond to a lower steady state concentration of butyryl–CoA. The total CoASH concentration in the matrix is 6–7 mM. The maximum uninhibited rate of β-oxidation of palmitoyl–carnitine is associated with low (0.3 mM) concentrations of CoASH in the matrix [41, 56], so it is clear that *extensive acylation of the available CoASH is necessary* to inhibit the remaining rate of β-oxidation.

serve as a weak buffer against accumulation of butyryl–CoA.

Inhibition of β-oxidation by metabolites of pent-4-enoate. Low concentrations (10–100 μM) of pent-4-enoate specifically inhibit β-oxidation in isolated mitochondria [6, 36]; however, much higher concentrations (often up to 1.7 mM) are required to cause severe metabolic disturbances in perfused livers and tissue homogenates [44, 45]. Other facts which must be considered are that pent-4-enoate does not always inhibit its own oxidation (see Fig. 3b), that it does not inhibit β-oxidation in soluble extracts, that no irreversible inhibition of any enzyme of β-oxidation has been found, and, contrary to earlier suggestions that only the oxidation of long-chain fatty acids is inhibited, that the β-oxidation of acyl–carnitines of all chain lengths is inhibited [6]. It is also clear that β-oxidation can be inhibited by 50 per cent in rat liver mitochondria by such low concentrations of pent-4-enoate (20 μM) that there is only a slight decrease in CoASH concentrations and that even in the presence of 0.1–1.0 mM pent-4-enoate, when β-oxidation is inhibited by 80–90 per cent, significant CoASH concentrations (0.1–0.3 mM) still occur in the matrix. We have shown that the inhibition of β-oxidation by pent-4-enoate and the inhibition of its own oxidation can be dissociated (see Fig. 3b). Similarly, the inhibition of β-oxidation can be dissociated from that of other CoA-requiring reactions. In our opinion, these results indicate that inhibition of β-oxidation is caused by low concentrations of a metabolite of pent-4-enoate whilst higher concentrations of pent-4-enoate cause a more extensive acylation of matrix CoASH, which also inhibits the self oxidation of pent-4-enoate as well as other CoA-requiring reactions.

A kinetic explanation of the effects of pent-4-enoate, both on β-oxidation and its own oxidation, together with the possible sites of inhibition, is outlined in Fig. 9 (see also [47]).

Mechanism of the inhibition of gluconeogenesis by hypoglycin and by pent-4-enoate

Two possible sites of inhibition of gluconeogenesis from pyruvate (or from lactate and alanine) have been identified in rat livers perfused with pent-4-enoate [44]. These are a block at the formation of triose phosphate by glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) thought due to a lack of NADH, and a block at pyruvate carboxylase thought due to a lack of acetyl–CoA [44]. The proposed deficiences of NADH and acetyl–CoA were assumed to be consequences of the inhibition of β-oxidation by pent-4-enoate. Indeed, the acetoacetate/3-hydroxybutyrate ratio, reflecting the redox state of the mitochondrial matrix, in the blood of animals given hypoglycin or pent-4-enoate [73, 74], or in isolated rat hepatocytes incubated with pent-4-enoate or MCPP [75], becomes more oxidized.

The data presented in this paper suggests that the block at pyruvate carboxylase is not simply caused by a deficiency of acetyl—CoA but that competitive inhibition of the allosteric activation of this enzyme by pent-4-enoyl–CoA, or by butyryl–CoA, isovaleryl–CoA and 2-methylbutyryl–CoA which accumulate in hypoglycin poisoning, is more important. Other unusual acyl–CoA esters derived from pent-4-enoate may also influence pyruvate carboxylase activity, although we do not think that acryloyl–CoA is involved [2, 46]. A more detailed discussion of the mechanism by which these compounds cause hypoglycaemia *in vivo* is presented in the following paper [15].

Acknowledgements—We thank Mrs. R. Seeley of the Department of Agricultural Biochemistry for the amino acid analyses and Dr. A. de Sa of the Department of Physics and Mr. A. Ward of this department for modifying the spectrofluorimeter. Mr. M. Barton provided excellent technical assistance. D.B. was in receipt of a Medical Research Council training grant and we are grateful to the Medical Research Council for financial support.

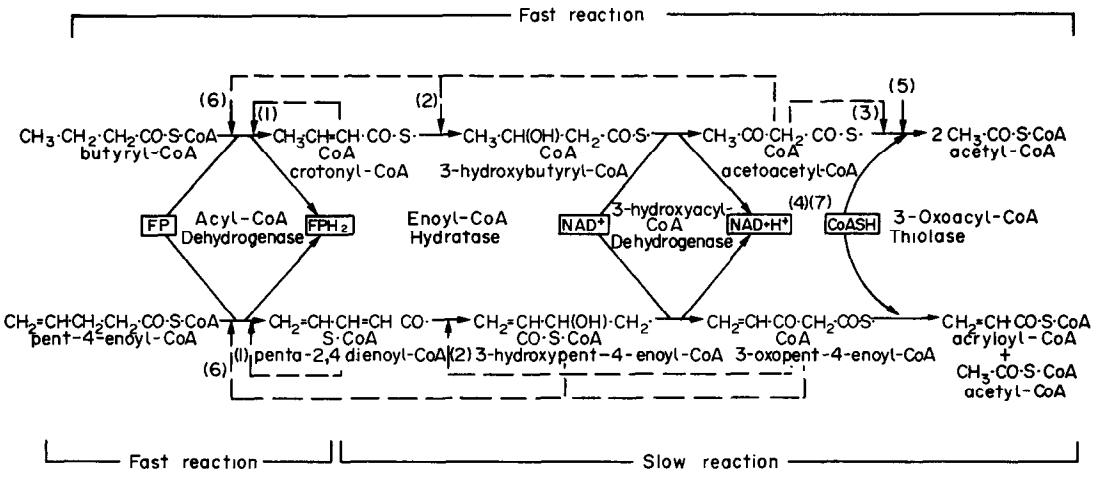

Fig. 9. Possible mechanisms for the inhibition of β-oxidation by pent-4-enoate. The upper line shows the rapid oxidation of butyryl–CoA to acetyl–CoA. The lower line shows the oxidation of pent-4-enoyl–CoA to acryloyl–CoA and acetyl–CoA; the first step of this conversion is rapid but the further metabolism of penta-2,4-dienoyl–CoA is slow [7]. It is proposed that the intermediates of pent-4-enoyl–CoA oxidation (penta-2,4-dienoyl–CoA, 3-hydroxypent-4-enoyl–CoA and 3-oxopent-4-enoyl–CoA) can inhibit normal β-oxidation as well as their own oxidation causing accumulation of other intermediates of β-oxidation, which can either exert feedback inhibition or further direct inhibition. The possible sites of feed back inhibition by intermediates of β-oxidation are: 1. Product inhibition of acyl–CoA dehydrogenases [70]. 2. Inhibition of enoyl–CoA hydratase by acetoacetyl–CoA [71]. 3. Substrate inhibition of 3-oxoacyl–CoA thiolase by acetoacetyl–CoA [72]. 4. Control of acyl–CoA dehydrogenase and of 3-hydroxyacyl–CoA dehydrogenase by the redox state of the matrix. 5. Inhibition of acetoacetyl–CoA thiolase by acetoacetyl–CoA. The possible sites of inhibition by metabolites of pent-4-enoate are: 1. Product inhibition of acyl–CoA dehydrogenases by penta-2,4-dienoyl–CoA. 2. Inhibition of enoyl–CoA hydratase by 3-oxopent-4-enoyl–CoA (by analogy with acetoacetyl–CoA). 5. Inhibition of acetoacetyl–CoA thiolase by penta-2,4-dienoyl–CoA [7]. 6. Inhibition of acyl–CoA dehydrogenase by 3-exopent-4-enoyl–CoA (by analogy with acetoacetyl–CoA), or, less likely, by 3-hydroxypent-4-enoyl–CoA. However, any molecular organisation of β-oxidation [50] could prevent some of these interactions. In addition, the intramitochondrial CoASH concentration (7) and energy charge (a high ATP/ADP ratio will favour a reduced state by slowing electron transport) will influence the rate of flux through these pathways (see [2] and [47]). A more complex model is needed for inhibition or palmitoyl–CoA oxidation when 7 cycles of β-oxidation are involved.

REFERENCES

1. H. S. A. Sherratt, *Br. med. Bull.* **25**, 250 (1969).
2. H. S. A. Sherratt and H. Osmundsen, *Biochem. Pharmac.* **25**, 743 (1976).
3. C. von Holt, *Biochim. biophys. Acta* **125**, 1 (1966).
4. H. V. Anderson, J. L. Johnson, J. W. Nelson, E. C. Alson, M. E. Spector and J. H. Vavra, *Chem. Inds, Lond.* 330 (1958).
5. R. Bressler, C. F. Corredor and K. Brendel, *Pharmac. Rev.* **21**, 105 (1969).
6. P. C. Holland and H. S. A. Sherratt, *Biochem. J.* **136**, 157 (1973).
7. P. C. Holland, A. E. Senior and H. S. A. Sherratt, *Biochem. J.* **136**, 172 (1973).
8. A. E. Senior and H. S. A. Sherratt, *Biochem. J.* **110**, 521 (1968).
9. D. Billington, H. Osmundsen, J. R. Taylor and H. S. A. Sherratt, *Biochem. Soc. Trans.* **4**, 1035 (1976).
10. H. Osmundsen, D. Billington, J. R. Taylor and H. S. A. Sherratt, *Biochem. J.* **170**, 337 (1978).
11. H. Osmundsen and H. S. A. Sherratt, *FEBS Lett.* **55**, 38 (1975).
12. K. Tanaka, E. M. Miller and K. J. Isselbacher, *Proc. natn. Acad. Sci. U.S.A.* **68**, 20 (1971).
13. K. Tanaka, K. J. Isselbacher and V. E. Shih, *Science* **175**, 69 (1972).
14. H. Osmundsen, D. Billington and H. S. A. Sherratt, *Biochem. Soc. Trans.* **2**, 1286 (1974).
15. D. Billington, H. Osmundsen and H. S. A. Sherratt, *Biochem. Pharmac.* **27**, 2891 (1978).
16. D. Billington, E. A. Kean, H. Osmundsen and H. S. A. Sherratt, *IRCS (Research on Biochemistry: Pharmacology)* **2**, 1712 (1974).
17. H. Osmundsen and H. S. A. Sherratt, *Biochem. Soc. Trans.* **3**, 330 (1975).
18. H. Osmundsen, D. Billington and H. S. A. Sherratt, *Biochem. Soc. Trans.* **3**, 331 (1975).
19. D. Billington, H. Osmundsen and H. S. A. Sherratt, *Biochem. Soc. Trans.* **4**, 102 (1976).
20. L. Fowden, in *A Symposium on Hypoglycin* (Ed. E. A. Kean), p. 11. Academic Press, New York (1976).
21. D. E. Abrahams and E. A. Kean, *West Indian med. J.* **18**, 147 (1969).
22. P. M. Scott, H. G. Botting, B. P. C. Kennedy and J. E. Knippel, *J. Food Sci.* **39**, 1057 (1974).
23. E. A. Kean, *J. Pharm. Pharmac.* **26**, 639 (1974).
24. H. Rosen, *Archs Biochem. Biophys.* **67**, 10 (1957).
25. A. G. Fincham, in *A Symposium on Hypoglycin* (Ed. E. A. Kean), p. 21. Academic Press, New York (1976).
26. E. J. Simon and D. Shemin, *J. Am. chem. Soc.* **75**, 2520 (1953).
27. H. Schulz, *J. biol. Chem.* **249**, 2704 (1974).
28. J. F. A. Chase and P. K. Tubbs, *Biochem. J.* **99**, 32 (1966).
29. F. Lipmann and L. C. Tuttle, *J. biol. Chem.* **159**, 21 (1945).
30. T. Weiland and L. Rueff, *Angew. Chem.* **65**, 186 (1953).
31. M. Hirashima, T. Hayakawa and M. Koike, *J. biol. Chem.* **242**, 902 (1967).

32. V. Massey, *Biochim. biophys. Acta* **38**, 447 (1960).
33. G. B. Warren and K. F. Tipton, *Biochem. J.* **139**, 297 (1974).
34. H. Osmundsen and M. V. Park, *Biochem. Soc. Trans.* **3**, 327 (1975).
35. A. B. Graham and M. V. Park, *J. Pharm. Pharmac.* **26**, 531 (1974).
36. A. E. Senior and H. S. A. Sherratt, *Biochem. J.* **110**, 499 (1968).
37. H. Beinert, *Meth. Enzym.* **5**, 546 (1962).
38. B. Chance and G. R. Williams, *Adv. Enzymol.* **17**, 65 (1956).
39. H. B. Stewart, P. K. Tubbs and K. K. Stanley, *Biochem. J.* **132**, 61 (1973).
40. J. R. Williamson and B. E. Corkey, *Meth. Enzym.* **13**, 454 (1969).
41. P. B. Garland, D. Sheperd and D. W. Yates, *Biochem. J.* **97**, 587 (1965).
42. O. H. Lowry, N. F. Roseborough, A. L. Farr and D. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
43. A. E. Senior, B. Robson and H. S. A. Sherratt, *Biochem. J.* **110**, 511 (1968).
44. J. R. Williamson, S. G. Rognstad and M. J. Peterson, *J. biol. Chem.* **245**, 3242 (1970).
45. C. Corredor, K. Brendel and R. Bressler, *J. biol. Chem.* **244**, 1212 (1969).
46. C. Corredor, K. Brendel and R. Bressler, *Proc. natn. Acad. Sci. U.S.A.* **58**, 2299 (1967).
47. H. S. A. Sherratt, P. C. Holland, H. Osmundsen and A. E. Senior, in *A Symposium on Hypoglycin* (Ed. E. A. Kean), p. 127. Academic Press, New York (1976).
48. C. von Holt, M. von Holt and H. Böhm, *Biochim. biophys. Acta* **125**, 11 (1966).
49. E. A. Kean, *Biochim. biophys. Acta* **422**, 8 (1976).
50. K. K. Stanley and P. K. Tubbs, *Biochem. J.* **150**, 77 (1975).
51. S. J. Patrick, *Can. J. Biochem Physiol.* **14**, 1163 (1964).
52. A. G. Dawson and F. J. R. Hird, *Archs Biochem. Biophys.* **127**, 622 (1968).
53. W. A. Johnson and J. L. Connelly, *Biochemistry* **10**, 1967 (1972).
54. H. E. Solberg and J. Bremer, *Biochim. biophys. Acta* **222**, 372 (1970).
55. J. Bremer and E. J. Davis, *Biochim. biophys. Acta* **528**, 269 (1978).
56. M. H. Fukami and J. R. Williamson, *J. biol. Chem.* **246**, 1206 (1971).
57. P. C. Holland, Ph.D. dissertation, University of Newcastle upon Tyne (1971).
58. S. J. Gatley and H. S. A. Sherratt, *Biochem. J.* **158**, 317 (1976).
59. J. Bremer, *Biochim. biophys. Acta* **116**, 1 (1966).
60. P. B. Garland, D. W. Yates and B. A. Haddock, *Biochem. J.* **119**, 553 (1970).
61. H. R. Mahler, S. J. Wakil and R. M. Bock, *J. biol. Chem.* **204**, 453 (1953).
62. J. Bar-Tana, G. Rose and B. Shapiro, *Biochem. J.* **109**, 269 (1968).
63. P. Mitchell and J. Moyle, *Eur. J. Biochem.* **7**, 471 (1969).
64. S. E. Knowles, I. G. Jarrett, O. H. Filsell and F. J. Ballard, *Biochem. J.* **142**, 401 (1974).
65. M. F. Utter, D. B. Keech and M. F. Scrutton, *Advan. Enzyme Regulation*, **2**, (1964).
66. S. J. Gatley and H. S. A. Sharratt, *Biochem. J.* **166**, 39 (1977).
67. K. Bartlett and D. Gompertz, *Biochem. Med.* **10**, 15 (1974).
68. J. F. A. Chase, *Biochem. J.* **104**, 510 (1967).
69. N. D. Costa and A. M. Snoswell, *Biochem. J.* **152**, 161 (1975).
70. J. G. Hauge, *J. Am. chem. Soc.* **78**, 5256 (1956).
71. R. M. Waterson and R. L. Holl, *J. biol. Chem.* **247**, 5258 (1972).
72. B. Middleton, *Biochem. J.* **132**, 312 (1973).
73. D. H. Williamson and M. B. Wilson, *Biochem. J.* **94**, 19c (1965).
74. A. E. Senior and H. S. A. Sherratt, *J. Pharm. Pharmac.* **21**, 85 (1969).
75. H. S. A. Sherratt and J. R. Williamson, unpublished work (1973).